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Cyclic Opioid Peptide Agonists and Antagonists Obtained Via Ring-Closing Metathesis

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The opioid peptide H-Tyr-c[D-Cys-Phe-Phe-Cys]NH_2 cyclized via a methylene dithiother is a potent and selective μ opioid agonist (Przydial M.J. *et al.*, J Peptide Res, 66, 2005, 255). Dicarba analogues of this peptide with Tyr, 2′,6′-dimethyltyrosine (Dmt), 3-[2,6-dimethyl-4-hydroxyphenyl]propanoic acid (Dhp) or (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid [(2*S*)-Mdp] in the 1-position were prepared. The peptides were synthesized on solid-phase by substituting D-allylglycine and (2*S*)-2-amino-5-hexenoic acid in position 2 and 5, respectively, followed by ring-closing metathesis. Mixtures of *cis* and *trans* isomers of the resulting olefinic peptides were obtained, and catalytic hydrogenation yielded the saturated $-CH_2-CH_2-$ bridged peptides. All six Tyr^1- and Dmt^1-dicarba analogues retained high μ and δ opioid agonist potency and showed only slight or no preference for μ over δ receptors. As expected, the six Dhp^1- and (2*S*)-Mdp^1-dicarba analogues turned out to be μ opioid antagonists but, surprisingly, displayed a range of different efficacies (agonism, partial agonism or antagonism) at the δ receptor. The obtained results indicate that the μ versus δ receptor selectivity and the efficacy at the δ receptor of these cyclic peptides depend on distinct conformational characteristics of the 15-membered peptide ring structure, which may affect the spatial positioning of the exocyclic residue and of the Phe^3 and Phe^4 side chains.

Key words: opioid activity profiles, opioid peptide analogues, opioid peptide SAR, peptide synthesis, ring-closing metathesis

Abbreviations: Aha, (2*S*)-2-amino-5-hexenoic acid; Allylgly, allylglycine, DAMGO, H-Tyr-D-Ala-Gly-N$^{\alpha}$MePhe-Gly-ol; Dhp, 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid; DIC, 1,3-diisopropylcarbodiimide; Dmt, 2′,6′-dimethyltyrosine; DPDPE, H-Tyr-c[D-Pen-Gly-Phe-D-Pen]OH; DPLPE, H-Tyr-c[D-Pen-Gly-Phe-Pen]OH; DSLET, H-Tyr-D-Ser-Gly-Phe-Leu-Thr-OH; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; JOM-13, H-Tyr-c[D-Cys-Phe-D-Pen]OH; (2*S*)-Mdp, 2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid; MVD, mouse vas deferens; RCM, ring-closing metathesis; TFA, trifluoroacetic acid; U69,593, (5α,7α,8β-(-)-*N*-methyl-*N*-[7-(pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide.

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The first cystine-containing cyclic enkephalin analogues, H-Tyr-c[-D-Cys-Gly-Phe-D-(or L)-Cys]NH_2 were synthesized independently by two groups (1,2) and were found to have high binding affinity for μ and δ opioid receptors. The corresponding peptides with a free C-terminal carboxyl group, H-Tyr-c[D-Cys-Gly-Phe-D(or L)-Cys]OH, showed subnanomolar δ opioid agonist potency and moderate δ receptor selectivity (3). Replacement of the two Cys residues in the latter peptides with penicillamine residues resulted in compounds with high δ receptor selectivity [H-Tyr-c[D-Pen-Gly-Phe-D-Pen]OH (DPDPE) and H-Tyr-c[D-Pen-Gly-Phe-Pen]OH (DPLPE)] (4). Subsequently, a dermorphin-derived cyclic tetrapeptide analogue containing the Phe residue in the 3-position of the peptide sequence, H-Tyr-c[D-Cys-Phe-Cys]NH_2, was reported to be a μ receptor-selective agonist (5). Substitution of D-Pen for Cys in the 4-position of the latter peptide and replacement of the C-terminal carboxamide function with a free carboxyl group led to a compound, H-Tyr-c[D-Cys-Phe-D-Pen]OH (JOM-13) which is almost as δ-selective as DPDPE and has 3.5-fold higher binding affinity for δ receptors (6). More recently, a pentapeptide containing a Phe residue in the 3- and 4-position, H-Tyr-c[D-Cys-Phe-Phe-Cys]NH_2-cyclized via a methylene dithioether, was described as a selective μ opioid receptor ligand with very high binding affinity (7).

Replacement of the disulphide bridge in cyclic opioid peptides with a bismethylene moiety ($-CH_2-CH_2-$) or a carbon–carbon double

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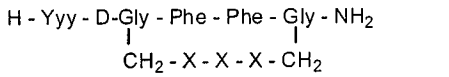
bond (–CH=CH–) is of interest because the resulting dicarba analogues have altered conformational properties and may show different opioid activity profiles. In the past, this structural modification was synthetically demanding, as it required the replacement of cystine with diaminosuberic acid in cumbersome multistep syntheses (8,9). The development of ring-closing metathesis (RCM) using Grubbs catalysts (10) has greatly facilitated the preparation of dicarba analogues of cystine-containing peptides, as exemplified by the recent synthesis of a dicarba analogue of oxytocin (11).

The first report on the replacement of the disulphide bond in cystine-containing opioid peptides with a carbon-carbon single- or double bond described the synthesis and characterization of the dicarba analogues of the cyclic tetrapeptides H-Tyr-c[D-Cys-Phe-Cys]NH_2 (5) and H-Tyr-c[D-Cys-Phe-D-Cys]NH_2 (12). This was achieved by substitution of allylglycine (Allylgly) for Cys in the 2- and 4-position and subsequent RCM, resulting in the cyclic olefinic peptides which upon catalytic hydrogenation yielded the saturated $-CH_2-CH_2-$ bridged peptide. The $-CH_2-CH_2-$ bridged peptide with L-configuration in position 4 showed about the same μ and δ opioid agonist potencies as its cystine-containing parent in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays, whereas the bismethylene analogue with D-configuration in position 4 was less potent than its parent by an order of magnitude. The cyclic olefinic peptides were also significantly less potent than their respective disulphide-bridged parents in both assays. Dicarba analogues of the enkephalin-derived cyclic peptides H-Tyr-c[D-Cys-Gly-Phe-D(or L)-Cys]NH_2 were first reported by Schiller's group (13,14). RCM of the Allylgly-substituted precursor peptides resulted again in mixtures of *cis* and *trans* isomers of the cyclic olefinic peptides which, upon catalytic hydrogenation, yielded the bismethylene analogues. All dicarba analogues retained high μ and δ opioid agonist potencies. Remarkably, the *trans* isomer of H-Tyr-[D-Allylgly-Gly-Phe-L-Allylgly]NH_2 was a μ agonist/δ agonist with subnanomolar potency at both receptors. Subsequently, the same dicarba analogues of H-Tyr-c[D-Cys-Gly-Phe-D-Cys]NH_2 with a C-terminal carboxylic acid group, H-Tyr-c[D-Cys-Gly-Phe-D-Cys]OH (3), were also reported to have high μ and δ opioid agonist activity (15).



no.	Yyy	- X - X - X -	
1	Tyr	- HC = CH - CH_2 -	*(cis)*
2	Tyr	- HC = CH - CH_2 -	*(trans)*
3	Tyr	- CH_2 - CH_2 - CH_2 -	
4	Dmt	- HC = CH - CH_2 -	*(cis)*
5	Dmt	- HC = CH - CH_2 -	*(trans)*
6	Dmt	- CH_2 - CH_2 - CH_2 -	
7	Dhp	- HC = CH - CH_2 -	*(cis)*
8	Dhp	- HC = CH - CH_2 -	*(trans)*
9	Dhp	- CH_2 - CH_2 - CH_2 -	
10	(2*S*)-Mdp	- HC = CH - CH_2 -	*(cis)*
11	(2*S*)-Mdp	- HC = CH - CH_2 -	*(trans)*
12	(2*S*)-Mdp	- CH_2 - CH_2 - CH_2 -	
13	Tyr	- S - CH_2 - S -	

Figure 1: Structural formulas of compounds **1–13.**

In the present paper, we describe the syntheses and in vitro opioid activities of dicarba analogues of the μ agonist peptide H-Tyr-c[D-Cys-Phe-Phe-Cys]NH_2 cyclized via a methylene dithioether (7) (Figure 1, compound **13**). The goal was to assess the effect of replacing the sulphurs of this peptide with methylenes on the opioid activity profile. As substitution of 2′,6′-dimethyltyrosine (Dmt) for Tyr^1 in opioid peptides is known to generally result in an opioid potency enhancement (16), the corresponding dicarba analogues with Dmt in place of Tyr^1 were also synthesized. Replacement of Tyr^1 in opioid peptides with 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp) or (2*S*)-2-methyl-3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid [(2*S*)-Mdp] has been shown to convert opioid agonist peptides into antagonists (17,18). In an effort to develop μ-selective opioid antagonists, we also prepared dicarba analogues of **13** containing Dhp or (2*S*)-Mdp in place of Tyr^1.

The syntheses were performed on solid phase (Rink amide Nova Gel resin) by assembly of the linear precursor peptides containing D-Allylgly and (2*S*)-2-amino-5-hexenoic acid (Aha) in position 2 and 5, respectively, and subsequent RCM using a second generation Grubbs catalyst. After cleavage from the resin, the olefinic peptides were obtained as mixtures of *cis* and *trans* isomers and subsequent catalytic hydrogenation yielded the saturated $-CH_2-CH_2-$ bridged peptides. Opioid activities of the compounds *in vitro* were determined in the GPI and MVD bioassays, and in μ-, δ- and κ-receptor binding assays.

Methods and Materials

General methods

Precoated plates (silica gel 60 F_{254}, 250 μm, Merck, Darmstadt, Germany) were used for ascending TLC in the following systems (all v/v); (I) *n*-BuOH/AcOH/H_2O (4:1:1); (II) *n*-BuOH/pyridine/AcOH/H_2O (15:10:3:12). Preparative reversed-phase HPLC was performed on a Vydac 218-TP1022 column (22 × 250 mm) with a linear gradient of 30–55% MeOH in 0.1% trifluoroacetic acid (TFA) over 40 min at a flow rate of 13 mL/min for the Tyr^1- and Dmt^1-peptide analogues, and with a linear gradient of 40–70% MeOH in 0.1% TFA over 50 min at the same flow rate for the Dhp^1- and (2*S*)-Mdp^1-peptide analogues. Analytical reversed-phase HPLC was performed on a Vydac 218-TP54 column (5 × 250 mm) at a flow rate of 1.5 mL/min using the same linear gradients of MeOH in 0.1% TFA as in the preparative HPLC. The same column was also used for the determination of the capacity factors (K′ values) under the same conditions. Molecular masses of the compounds were determined by electrospray mass spectrometry on a Hybrid Q-Tof mass spectrometer interfaced to a MassLynx 4.0 data system (Micromass Ltd., Pointe-Claire, QC, Canada).

Peptide synthesis

The linear peptides were prepared by the manual solid-phase technique using Fmoc-protection for the α-amino group of amino acids and 1,3-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) as coupling agents. The peptides were assembled on a Rink amide resin (Novabiochem, Läufelfingen, Switzerland) according to a published protocol (19). After completion of peptide assembly, cyclization

between the Allylgly and 2-amino-5-hexenoic acid residues was achieved using the second generation Grubbs catalyst benzylidene[1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene]dichloro(tricyclohexylphosphine)-ruthenium (20). The catalyst in 20 mol% (as compared with resin-bound peptide) was added to the peptide resin suspended in CH_2Cl_2 under a flow of argon. The reaction mixture was refluxed for 48 h, and after cooling to room temperature, dimethylsulfoxide (DMSO) (50 equiv relative to the catalyst) was injected and the mixture was stirred for another 24 h. The peptide resin was filtered and washed successively with DMSO, CH_2Cl_2 and MeOH. Fmoc protection was removed by 30% piperidine/*N,N*-dimethylformamide (DMF) treatment, and the cyclic olefinic peptides were cleaved from the resin by TFA treatment in the usual manner. The yield of the cyclization reaction was 40–47% for the Tyr^1-, Dmt^1- and (2*S*)-Mdp^1-analogues and 89% for the Dhp peptide. Mixtures of *cis* and *trans* isomers were obtained in all cases and the configuration of the double bond was established by measurement of the *J* coupling constants between the olefinic protons (*cis*: *J* ~10 Hz; *trans*: *J* ~15 Hz). The *cis/trans* ratios for the peptides containing the four different N-terminal residues were as follows: Tyr (3:1), Dmt (6.7:1), Dhp (1.4:1), (2*S*)-Mdp (2:1). The crude peptides were purified by preparative reversed-phase HPLC and were found to be at least 98% pure, as assessed by HPLC and TLC. Catalytic hydrogenation of the cyclic olefinic peptides was performed with the mixtures of the *cis* and *trans* isomers with 10% Pd/C in EtOH at 40°C for 18 h (p_{H2} = 45 psig). The resulting $-CH_2-CH_2-$ bridged peptides were obtained in 75–98% yield and were purified by preparative HPLC.

H-Tyr-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*cis*; 1)

HPLC *K'* 1.236; TLC R_f 0.742 (I), R_f 0.782 (II); ES-ML *m/e* 655; ^{1}H NMR (500 MHz, CD_3OD) δ 7.36-7.28 (m, 4H), 7.28-7.15 (m, 4H), 7.15-7.07 (d, 2H, *J* = 8.5 Hz), 7.01-6.97 (d, 2H, *J* = 8.5 Hz), 6.83-6.79 (d, 2H, *J* = 8.5 Hz), 5.41-5.34 (ddd, 1H, *J* = 6.5 Hz, *J* = 6.5 Hz, *J* = 10.7 Hz), 5.27-5.20 (ddd, 1H, *J* = 3.0 Hz, *J* = 10.7 Hz, *J* = 11.9 Hz), 4.43-4.39 (dd, 1H, *J* = 6.2 Hz, *J* = 8.3 Hz,), 4.39-4.33 (dd, 1H, *J* = 3.4 Hz, *J* = 10.7 Hz), 4.22-4.17 (dd, 1H, *J* = 4.15 Hz, *J* = 8.3 Hz), 4.13-4.05 (m, 2H), 3.30-3.23 (m, 1H), 3.23-3.17 (m, 1H), 3.12-2.98 (m, 4H), 2.90 (s, 2H), 2.90-2.83 (m, 2H), 2.40-2.31 (m, 1H), 2.31-2.22 (m, 1H), 2.08-2.02 (m, 1H), 1.98-1.93 (m, 1H), 1.80-1.72 (m, 1H), 1.37-1.31 (m, 2H).

H-Tyr-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*trans*; 2)

HPLC *K'* 1.270; TLC R_f 0.730 (I), R_f 0.772 (II); ES-ML *m/e* 655; ^{1}H NMR (500 MHz, CD_3OD) δ 7.40-7.28 (m, 5H), 7.28-7.18 (m, 3H), 7.13-7.11 (d, 2H, *J* = 8.5 Hz), 7.10-7.08 (d, 2H, *J* = 8.5 Hz), 6.83-6.81 (d, 2H, *J* = 8.5 Hz), 5.14-5.04 (m, 2H), 4.64-4.60 (dd, 1H, *J* = 6.2 Hz, *J* = 8.3 Hz), 4.34-4.30 (dd, 1H, *J* = 3.4 Hz, *J* = 10.7 Hz), 4.30-4.25 (dd, 1H, *J* = 4.15 Hz, *J* = 8.3 Hz), 4.21-4.17 (t, 1H, *J* = 7.8 Hz), 4.18-4.15 (t, 1H, *J* = 7.8 Hz), 3.22-3.00 (m, 7H), 2.90 (s, 2H), 2.40-2.34 (m, 1H), 2.10-1.90 (m, 4H), 1.80-1.72 (m, 1H).

H-Tyr-c[D-Allylgly-Phe-Phe-Aha]NH_2 (saturated; 3)

HPLC *K'* 1.260; TLC R_f 0.659 (I), R_f 0.765 (II); ES-ML *m/e* 657; ^{1}H NMR (500 MHz, CD_3OD) δ 7.38-7.14 (m,10H), 7.08-7.06 (d, 2H, *J* = 8.5 Hz), 6.84-6.82 (d, 2H, *J* = 8.5 Hz), 4.75-4.71 (dd, 1H, *J* = 6.2 Hz, *J* = 8.3 Hz), 4.38-4.34 (dd, 1H, *J* = 4.15 Hz, *J* = 8.3 Hz), 4.28-4.24 (dd, 1H, *J* = 3.4 Hz, *J* = 10.7 Hz), 4.20-4.14 (m, 2H), 3.40-3.20 (m, 6H),2.05-1.90 (m, 2H), 1.70-1.55 (m, 2H), 1.40-1.20 (m, 6H).

H-Dmt-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*cis*; 4)

HPLC *K'*1.211; TLC R_f 0.840 (I), R_f 0.838 (II); ES-ML *m/e* 683; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.80-9.70 (s, 1H), 8.51-8.42 (s, 2H), 8.30-8.25 (d, 1H, *J* = 8.5 Hz), 8.05-8.00 (d, 1H, *J* = 8.5 Hz), 7.90-7.85 (d, 1H, *J* = 8.5 Hz), 7.40-7.15 (m, 10H), 7.08-7.05 (s, 2H), 6.55-6.52 (s, 2H), 5.36-5.30 (m, 1H), 5.14-5.06 (m, 1H), 4.25-4.12 (m, 3H), 4.06-4.00 (m, 1H), 3.83-3.75 (m, 1H), 3.26-3.20 (m, 2H), 3.09-2.96 (m, 4H), 2.90-2.83 (m, 2H), 2.28-2.24 (s, 6H), 2.07-2.00 (m, 1H), 1.85-1.74 (m, 1H).

H-Dmt-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*trans*; 5)

HPLC *K'* 1.230; TLC R_f 0.824 (I), R_f 0.842 (II); ES-ML *m/e* 683; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.28-9.22 (s, 1H), 8.68-8.56 (d, 3H, *J* = 8.5Hz), 8.18-8.14 (d, 1H, *J* = 8.5 Hz), 7.96-7.90 (d, 2H, *J* = 8.5 Hz), 7.38-7.16 (m, 9H), 7.10-7.04 (m, 2H), 6.68-6.64 (s, 1H), 6.62-6.58 (s, 2H), 5.06-4.98 (ddd, 1H, *J* = 4.9 Hz, *J* = 9.05 Hz, *J* = 14.1 Hz), 4.90-4.82 (ddd, 1H, *J* = 6.95 Hz, *J* = 7.3 Hz, *J* = 14.1 Hz), 4.55-4.48 (dt, 1H, *J* = 5.3 Hz, *J* = 8.8 Hz), 4.25-4.20 (dt, 1H, *J* = 4.4 Hz, *J* = 8.0 Hz), 4.20-4.12 (m, 2H), 4.02-3.93 (m, 1H), 3.18-2.89 (m, 6H), 2.83-2.80 (d, 1H, *J* = 4.1Hz), 2.26-2.22 (s, 6H), 2.26-2.16 (m, 1H), 2.10-1.88 (m, 3H), 1.75-1.67 (m, 1H), 1.62-1.53 (m, 1H).

H-Dmt-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*saturated*; 6)

HPLC *K'* 1.220; TLC R_f 0.791 (I), R_f 0.815 (II); ES-ML *m/e* 685; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.40-7.03 (m, 10H), 6.62-6.58 (s, 2H), 4.75-4.71 (dd, 1H, *J* = 6.2 Hz, *J* = 8.3 Hz), 4.38-4.34 (dd, 1H, *J* = 4.15 Hz, *J* = 8.3 Hz), 4.28-4.24 (dd, 1H, *J* = 3.4 Hz, *J* = 10.7 Hz), 4.22-4.14 (m, 2H), 3.28-3.02 (m, 6H), 2.30-2.22 (s, 6H), 2.05-1.90 (m, 2H), 1.75-1.60 (m, 2H), 1.40-1.23 (m, 6H).

Dhp-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*cis*; 7)

HPLC *K'* 1.181; TLC R_f 0.912 (I), R_f 0.891 (II); ES-ML *m/e* 668; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.63-8.59 (d, 1H, *J* = 8.05 Hz), 8.52-8.47 (d, 1H, *J* = 8.78 Hz,), 8.22-8.18 (d, 1H, *J* = 6.84 Hz), 7.49-7.46 (d, 1H, *J* = 6.1Hz), 7.43-7.40 (s, 1H), 7.36-7.23 (m, 12H), 7.20-7.17 (m, 1H), 5.48-5.36 (m, 2H), 4.96-4.90 (m, 2H), 4.74-4.64 (dd, 2H, *J* = 4.15 Hz, *J* = 3.42 Hz, *J* = 10.1 Hz, *J* = 10.7 Hz), 4.38-4.20 (m, 1H), 4.09-4.03 (m, 1H), 3.15-3.07 (m, 2H), 3.00-2.82 (m, 8H), 2.76-2.67 (m, 2H), 2.28-2.18 (m, 2H), 2.11-2.00 (m, 2H), 1.81-1.70 (m, 1H), 1.68-1.57 (m, 1H).

Dhp-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*trans*; 8)

HPLC *K'* 1.162; TLC R_f 0.901 (I), R_f 0.875 (II); ES-ML *m/e* 668; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.00 s, 1H), 8.11-8.06 (d, 1H, *J* = 7.8 Hz), 8.02-7.98 (d, 1H, *J* = 8.8 Hz), 7.90-7.86 (d, 1H, *J* = 6.6 Hz), 7.81-7.76 (d, 1H, *J* = 8.1 Hz), 7.38-7.14 (m, 10H), 7.10-7.05 (s, 1H), 6.72-6.68 (s, 1H), 6.65-6.60 (s, 2H), 5.26-5.19 (ddd, 1H,

J = 5.6 Hz, J = 8.5 Hz, J = 14.7 Hz), 5.13-5.06 (ddd, 1H, J = 6.6 Hz, J = 7.8 Hz, J = 14.7 Hz), 4.60-4.52 (m, 1H), 4.25-4.13 (m, 2H), 4.05-3.95 (m, 1H), 3.12-2.86 (m, 4H), 2.72-2.65 (m, 4H), 2.42-2.28 (m, 2H), 2.28-2.24 (s, 6H), 2.08-2.00 (m, 2H), 1.94-1.86 (m, 1H).

Dhp-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*saturated*; 9)

HPLC K' 1.156; TLC R_f 0.791 (I), R_f 0.853 (II); ES-ML m/e 670; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.00 (s, 1H), 8.14-8.10 (d, 1H, J = 7.8 Hz), 8.05-8.01 (d, 1H, J = 8.8 Hz), 7.92-7.90 (d, 1H, J = 6.6 Hz), 7.84-7.80 (d, 1H, J = 8.1 Hz), 7.40-7.16 (m, 10H), 7.11-7.06 (s, 1H), 6.74-6.70 (s, 1H), 6.65-6.60 (s, 2H), 4.62-4.54 (m, 1H), 4.28-4.18 (m, 1H), 4.18-4.12 (m, 1H), 4.00-3.94 (m, 1H), 3.15-2.85 (m, 4H), 2.75-2.66 (m, 4H), 2.30-2.22 (s, 6H), 1.90-1.75 (m, 2H), 1.65-1.50 (m, 2H), 1.42-1.22 (m, 6H).

(2*S*)-Mdp-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*cis*; 10)

HPLC K' 1.138; TLC R_f 0.923 (I), R_f 0.902 (II); ES-ML m/e 682; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.95 (s, 1H), 8.21-8.18 (d, 1H, J = 7.3 Hz), 7.58-7.55 (d, 1H, J = 7.8 Hz), 7.45-7.40 (d, 1H, J = 8.1 Hz), 7.35-7.30 (d, 1H, J = 8.5 Hz), 7.30-6.98 (m, 12H), 6.41 (s, 2H), 5.28-5.21 (ddd, 1H, J = 4.3 Hz, J = 7.4 Hz, J = 11.7 Hz), 5.19-5.13 (ddd, 1H, J = 5.6 Hz, J = 9.5 Hz, J = 10.7 Hz), 4.24-4.10 (m, 3H), 3.87-3.81 (m, 1H), 3.12-3.02 (m, 3H), 2.72-2.54 (m, 4H), 2.16 (s, 6H), 2.04-1.93 (m, 2H), 1.79-1.68 (m, 3H), 1.48-1.40 (m, 1H), 0.98-0.96 (d, 3H, J = 6.4 Hz).

(2*S*)-Mdp-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*trans*; 11)

HPLC K' 1.108; TLC R_f 0.901 (I), R_f 0.897 (II); ES-ML m/e 682; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.00-9.85 (s, 1H), 8.03-7.98 (d, 1H, J = 8.5 Hz), 7.84-7.79 (d, 1H, J = 8.0 Hz), 7.67-7.62 (d, 1H, J = 8.0 Hz), 7.30-7.06 (m, 11H), 7.00 (s, 1H), 6.62 (s, 1H), 6.42 (s, 2H), 5.14-5.06 (ddd, 1H, J = 4.7 Hz, J = 9.0 Hz, J = 15.1 Hz), 4.97-4.90 (dt, 1H, J = 6.9 Hz, J = 15.1 Hz), 4.45-4.42 (m, 1H), 4.16-4.06 (m, 2H), 3.93-3.86 (m, 1H), 3.10-3.00 (m, 2H), 2.99-2.80 (m, 3H), 2.73-2.62 (m, 2H), 2.21-2.08 (m, 1H), 2.16 (s, 6H), 2.05-1.88 (m, 3H), 1.88-1.78 (m, 1H), 1.56-1.48 (m, 1H), 0.96-0.92 (d, 3H, J = 6.35 Hz).

(2*S*)-Mdp-c[D-Allylgly-Phe-Phe-Aha]NH_2 (*saturated*; 12)

HPLC K' 1.128; TLC R_f 0.890 (I), R_f 0.880 (II); ES-ML m/e 684; ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.00 (s,1H), 7.90-7.86 (d, 1H, J = 7.6 Hz), 7.85-7.82 (d, 1H, J = 7.6 Hz), 7.76-7.73 (d, 1H, J = 6.8 Hz), 7.40-7.20 (m, 11H), 7.15 (s, 2H), 6.48 (s, 2H), 4.50-4.41 (m, 1H), 4.38-4.33 (m, 1H), 4.26-4.21 (m, 1H), 3.92-3.86 (m, 1H), 3.16-2.98 (m, 6H), 2.26-2.20 (s, 6H), 1.24-1.22 (d, 3H, J = 8.3Hz),1.78-1.14 (m,11H).

In vitro bioassays and receptor binding assays

The GPI (21) and MVD (22) bioassays were carried out as reported in detail elsewhere (23,24). A dose–response curve was determined with [Leu^5]enkephalin as standard for each ileum and vas preparation, and IC_{50} values of the compounds being tested were normalized according to a published procedure (25). K_e values for antagonists were determined from the ratio of IC_{50} values obtained with an agonist in the presence and absence of a fixed antagonist concentration (26). μ Antagonist K_e values of compounds were determined in the GPI assay against the μ agonist H-Tyr-D-Ala-Phe-Phe-NH_2 TAPP (27) and δ antagonist K_e values were measured in the MVD assay against the δ agonist DPDPE. Opioid receptor binding studies were performed as described in detail elsewhere (23). Binding affinities for μ and δ receptors were determined by displacing, respectively, [^{3}H]DAMGO (Multiple Peptide Systems, San Diego, CA, USA) and [^{3}H]DSLET; (Multiple Peptide Systems) from rat brain membrane binding sites, and κ-opioid receptor binding affinities were measured by displacement of [^{3}H]U69,593 (Amersham) from guinea pig brain membrane binding sites. Incubations were performed for 2h at 0°C with [^{3}H]DAMGO, [^{3}H]DSLET and [^{3}H]U69,593 at respective concentrations of 0.72, 0.78 and 0.80 nM. IC_{50} values were determined from log-dose displacement curves, and K_i values were calculated from the obtained IC_{50} values by means of the equation of Cheng and Prusoff (28), using values of 1.3, 2.6 and 2.9 nM for the dissociation constants of [^{3}H]DAMGO, [^{3}H]DSLET and [^{3}H]U69,593, respectively.

Results

Nearly all Tyr^1- and Dmt^1-olefinic and saturated cyclic peptides (compounds **1–6**) showed subnanomolar μ and δ opioid agonist potencies in the functional GPI and MVD bioassays (Table 1). Most remarkable were the Dmt^1-analogues **5** and **6** which displayed picomolar δ agonist potencies in the MVD assay. In agreement with the functional assay data, agonist peptides **1–6** also showed subnanomolar or very low nanomolar μ and δ opioid receptor affinities in the binding assays, with the *cis* and *trans* olefinic peptides having slightly higher binding affinities than the corresponding saturated ones (Table 2). The obtained K_i^μ/K_i^δ ratios indicate that the Tyr^1-analogues **1–3** show only slight preference for μ over δ receptors, whereas the Dmt^1-analogues **4–6** are non-selective in their

Table 1: GPI and MVD assay of dicarba analogues of **13**[a]

	GPI		MVD	
Compound	IC_{50} (nM)	K_e (nM)[b]	IC_{50} (nM)	K_e (nM)[c]
1	0.401 ± 0.058		0.851 ± 0.057	
2	1.34 ± 0.18		4.51 ± 0.61	
3	1.05 ± 0.22		0.740 ± 0.043	
4	0.161 ± 0.017		0.191 ± 0.041	
5	0.407 ± 0.056		0.0444 ± 0.0076	
6	0.509 ± 0.021		0.0655 ± 0.0098	
7		32.2 ± 2.2	PA (e = 0.38)	
8		44.3 ± 3.5	239 ± 10	
9		41.7 ± 8.1	7500 ± 1630	
10		32.4 ± 5.7	PA	
11		157 ± 28	PA (e = 0.33)	
12		25.7 ± 1.1		147 ± 36

PA, partial agonist.

[a]Values represent means of 3–6 determinations ± SEM. [b]Determined against H-Tyr-D-Ala-Phe-Phe-NH_2 (TAPP). [c]Determined against DPDPE.

Table 2: Opioid receptor binding data of dicarba analogues of **13**

Compound	K_i (nM)[a]			K_i ratio
	μ[b]	δ[b]	κ[c]	$\mu/\delta/\kappa$
1	0.281 ± 0.028	0.910 ± 0.349	9.36 ± 2.73	1/3/12
2	0.332 ± 0.038	1.53 ± 0.89	15.2 ± 3.2	1/5/14
3	1.04 ± 0.14	2.24 ± 0.15	11.3 ± 2.6	1/2/11
4	0.528 ± 0.005	0.491 ± 0.011	12.8 ± 2.5	1/1/2
5	0.438 ± 0.012	0.286 ± 0.021	4.97 ± 1.18	1/1/11
6	1.54 ± 0.27	1.20 ± 0.14	15.8 ± 1.6	1/1/10
7	23.5 ± 3.9	86.3 ± 7.7	266 ± 51	1/4/11
8	16.7 ± 1.7	27.0 ± 0.3	>500	1/2/ > 30
9	8.95 ± 0.27	39.2 ± 3.6	>500	1/4/ > 56
10	7.44 ± 1.21	7.67 ± 0.87	185 ± 21	1/1/25
11	323 ± 36	35.3 ± 1.3	635 ± 73	1/0.1/2
12	18.3 ± 0.9	18.7 ± 1.7	302 ± 14	1/1/17
13[d]	0.016 ± 0.01	1.8 ± 0.8	2.5 ± 1.5	1/112/156

[a]Values represent mean of 3–6 determinations ± SEM. [b]Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DSLET (δ-selective) from rat brain membrane binding sites. [c]Displacement of [^{3}H]U69,593 (κ-selective) from guinea pig brain membrane binding sites. [d]Data taken from Przydzial *et al.* (7).

interaction with μ and δ opioid receptors. All six agonist peptides had relatively weaker binding affinity for κ opioid receptors.

As expected, the Dhp^1- and (2*S*)-Mdp^1-olefinic and saturated peptides (**7–12**) were quite potent μ opioid antagonists with K_e values ranging from 26 to 44 nM, except for the *trans* olefinic (2*S*)-Mdp^1-analogue (**11**) which had weaker μ antagonist activity (K_e^{μ} = 157 nM). In the MVD assay, these peptides were partial δ agonists (compounds **7**, **10** and **11**), weak full δ agonists (compounds **8** and **9**) or, in one case, a δ antagonist (**12**). Interestingly, compounds **8** and **9**, both lacking a positively charged nitrogen, are mixed δ agonist/μ antagonists. The μ receptor binding affinities of peptides **7–12** are in reasonable correlation with their μ agonist potencies determined in the GPI functional assay. Compounds **7–10** and **12** displayed either only slight or no preference for μ over δ receptors. In agreement with its weak μ opioid agonist potency in the GPI assay, the *trans* olefinic peptide **11** also displayed weak μ receptor affinity in the binding assay. Surprisingly, these data indicate that compound **11** is a δ receptor-selective ligand. Peptides **7–12** all showed very weak κ-receptor binding affinity.

Conclusions

The six Tyr^1- and Dmt^1-peptides (compounds **1–6**) all display similar μ and δ opioid agonist potencies despite the somewhat different conformational constraints in their ring structures, with the rings containing the –CH=CH– bond (*cis* or *trans*) being more rigid than those containing the $-CH_2-CH_2-$ bond. The parent peptide **13**, containing the methylene dithioether linkage, has been reported to be a very selective μ receptor ligand (K_e^{μ}/K_i^{δ} = 112) (7). In comparison with **13**, the Tyr^1- and Dmt^1-dicarba analogues with agonist properties described here show similar δ receptor binding affinity, but 18- to 96-fold lower μ receptor binding affinity. Consequently, these compounds have either no or only slight μ versus δ receptor selectivity. The very high μ receptor binding affinity of parent peptide **13** may be the result of specific conformational constraints in the peptide ring structure because of the presence of the two sulphur atoms.

As expected, compounds **7–12** are all antagonists at the μ opioid receptor. Compound **11** has 14- to 43-fold lower μ receptor binding affinity than the other Dhp^1- and (2*S*)-Mdp^1-analogues, possibly as a consequence of an unfavourable interaction of its (2*S*)-Mdp residue with the μ receptor in its inactive state. Unexpectedly, compounds **7–12** display a range of different efficacies (agonism, partial agonism or antagonism) at the δ receptor. This divergent behaviour may be due to the various conformational constraints in their peptide ring structures in conjunction with possible distinct intramolecular interactions of the extracyclic Dhp^1- or (2*S*)-Mdp^1-residues or distinct positioning of the Phe^3/Phe^4 side chains, which may affect their binding to the δ receptor in its activated or inactive state. Compounds **8** and **9** are rare examples of compounds with δ opioid *agonist* activity that lack a positively charged nitrogen.

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